Equid milk: Chemical and Physico-chemical properties

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THE HORSE

Among the most important domesticated animals, found worldwide.

Has played a major role in the development of human civilization.

- Military
- Transport
- Agriculture

In developed countries today –

- Mainly sporting activities (various)
- In some countries

  Meat

  Milk

DAIRY HORSES

- Important in Mongolia, Central Asian Steppes, Russia

  also: France, Italy, Hungary, Netherlands, etc.

- Number of dairy horses and amount of mares' milk produced not known precisely.

- Milk from 230,000 mares used in Russia for Koumiss

- ca 1 million kg equine milk produced in Europe (exl Russia) and ca 10 million kg in Asia

Dairy Horse Farm in Netherlands, Orchids, Zealand

GROSS COMPOSITION (%) OF EQUID MILK

	Total Solids	**Fat**	**Protein**	**Lactose**	**Ash**
Horse	10.4	1.4	1.82	6.74	0.47
Ass	10.8	1.8	1.74	5.87	0.44
Zebra	11.3	2.2	1.63	7.0	0.38
Cow	12.7	3.6	3.2	4.8	0.8
Human	12.3	3.6	1.4	6.7	0.3

Features: low fat, low protein, high lactose

Equid Milk

- Milk of all equids quite similar
- Equine milk fairly well characterized
- Quite a lot of information on asinine milk
- Few data on zebra milk – difficult to handle

MILK SUGARS

- Lactose principal sugar in milk of most eutherians but all contains oligosaccharides.
- OSs high concentration in human milk (> 15 g/L; > 130 oligosaccharides) also high in elephant and bear milk.
- Monotremes and marsupials: very little lactose, mainly oligosaccharides
- Equine milk, low level of OSs
- Much interest in significance of OSs

Fatty Acid Composition of Equine Milk

Fatty acid	% w/w*
$C_{4:0}$	< 1
$C_{6:0}$	< 1.5
$C_{8:0}$	2.0-6.1
$C_{10:0}$	2.3-16.7
$C_{12:0}$	3.8-14.6
$C_{14:0}$	5.3-10.7
$C_{14:1}$	0.1-2.6
$C_{16:0}$	12.4-23.8
$C_{16:1}$	2.2-9.7
$C_{18:0}$	0.0-3.0
$C_{18:1}$	9.4-28.2
$C_{18:2}$	3.6-17.9
$C_{18:3}$	1.5-26.2

Notable features:
Very high C10:0 and C12:0
High PUFA

Ratio of ω:6:ω:3 is 1.16:1 in asinine and 1.26:1 in equine milk – optimum for reduction of risk of cardiovascular disease & some cancers (bovine milk ~ 2-3:1)

*(extreme variations from 18 publications)

FAT GLOBULES

<1 – 5 μm in diameter;

mean 2.5-3 μm

- Slightly smaller than bovine milk fat globules
- No creaming (no cryoglobulin)

Human and Equine Milk Fat Globules

Filamentous surface structure

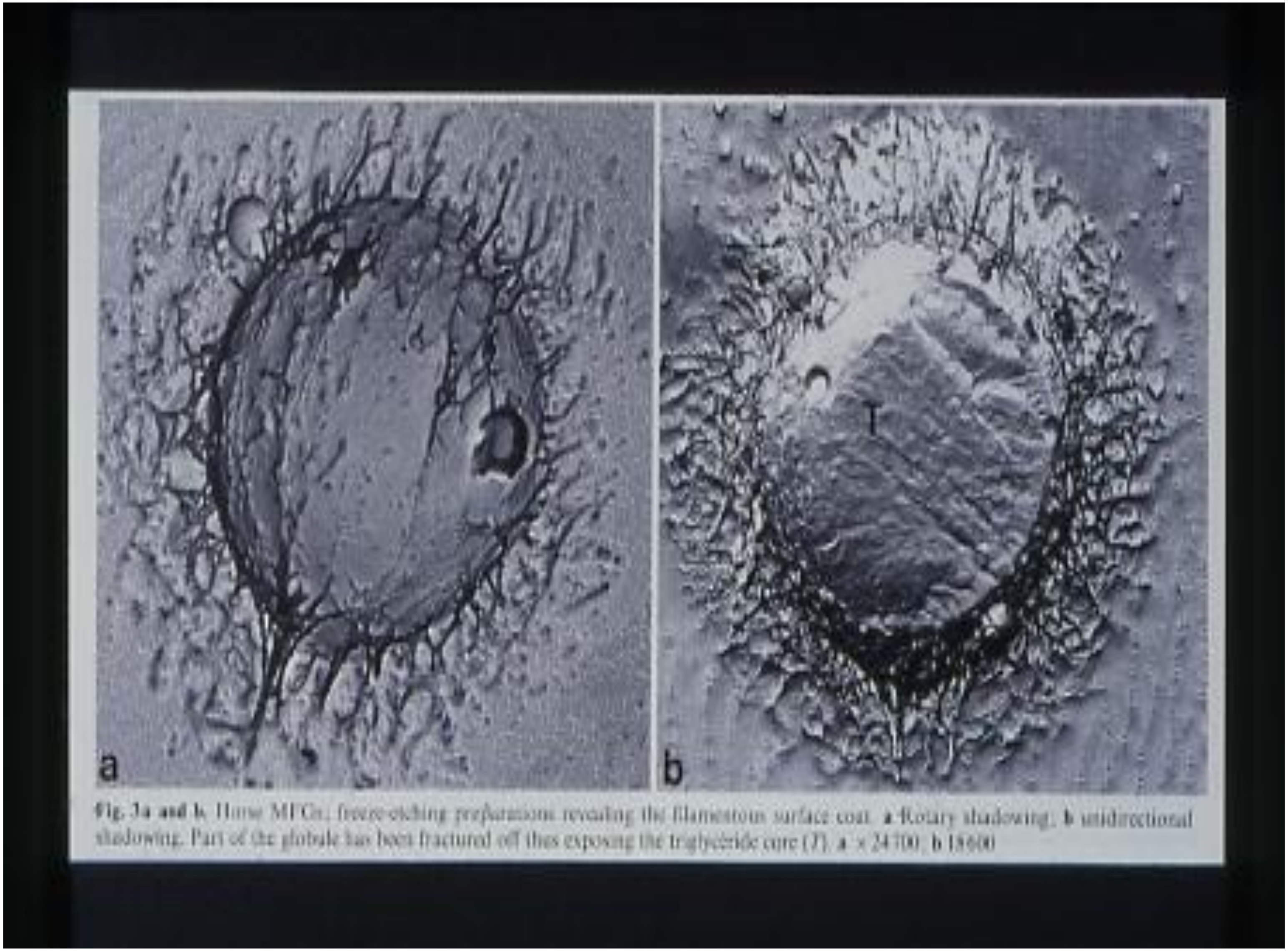
High MW glycoproteins (mucins)→ shed on heating

Facilitate fat absorption

→ adherence to gut mucus retards globule movement

→ inactivation of milk lipase

EXISTING SLIDES OF EQUINE MFG



TYPES OF PROTEINS (g/kg) IN EQUINE MILK

	Human	Mare	Cow
Total Protein	14.2	21.4	32.5
Casein	3.7	10.7	25.1
Whey Proteins	7.6	8.3	5.7
Cas: NCN	1:2.0	1:0.8	1:0.2
NPN	20.4	11.2	5.2
β-Lg (% of WP)	0	30.7	53.6
α-La (% of WP)	42.4	28.6	20.1
BSA (% of WP)	7.7	4.5	6.2
Ig (% of WP)	18.2	19.8	11.8
Lf	30.3	9.9	8.4
Lysozyme	1.7	6.6	Tr

EQUINE α - LACTALBUMIN

Equine milk: approx equal amounts of α-La and β-Lg

Equine and asinine α-La- generally similar to other α-La’s

Small molecule: 123 AA residues; MW: 14,215 Da

4 intra-molecular disulphides

Binds Ca^{2+} in a positive loop

Genetic variants, A (principal),B, C, differ in amino acid profile

2 isoforms of α-La A differing in degree of amidation or glycosylation

Amino acid sequence known (Giradet *et al* IDJ 14 207-217 (2004).

α-Lactalbumin

- Biological function:
- Modifies specificity of UDP galactosyl transferase in lactose synthesis
- Makes it highly specific for glucose
- Reduces Km 1000 fold
- Concentration of lactose in milk proportional to concentration of α-La
  - both absent from milk of some marine mammals

β-Lactoglobulin

- Occurs in the milk of most species
  → exceptions - human, most primates, rodents
- β-Lg of some species has a sulphydryl group, others do not
- Some dimerize, others do not
- Dimerization and –SH not related

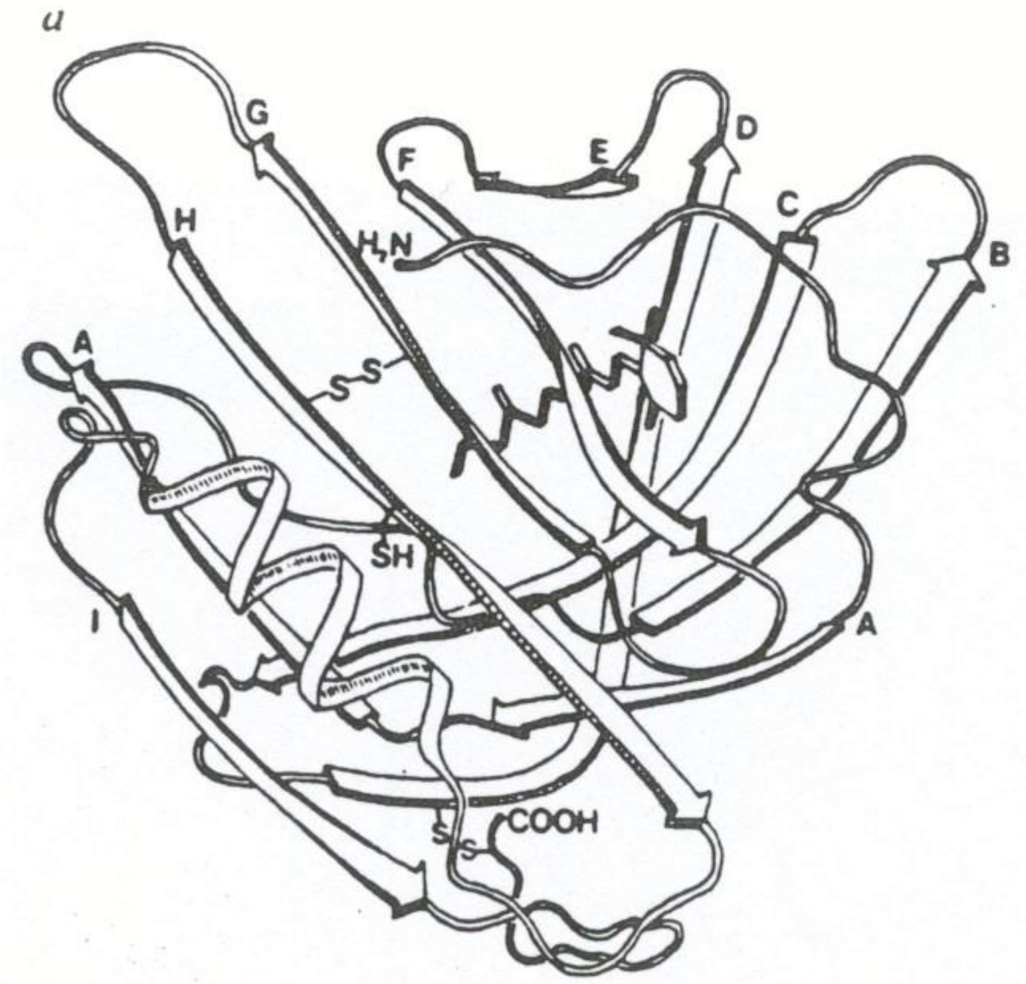
Bovine β-Lactoglobulin

- Highly structured small molecule
  - 162 residues
  - 18.3 kDa
- 2 intra-molecular disulphides
- 1 sulphydryl group
- Amino acid sequence known

- Spherical, 0.36 nm in diameter

- Quaternary structure:
  - Monomeric <pH 3.5 > 7.5
  - Dimeric pH 5.5-7.5
  - Octameric pH 3.5-5.5

Secondary and Tertiary Structures of Bovine β-Lactoglobulin



β-Lactoglobulin

- Biological function
  - Bind hydrophobic molecules in a hydrophobic pocket
  - Two possible functions:

➢ Binds and protects retinol against oxidation;

- exchanges with a retinol-binding protein in intestine
- Questions:
- Where does exchange of retinol from fat globule to β-Lg occur?
- Why do humans and rodents not have β-Lg

➢ Binds fatty acids → promotes lipase activity

β-Lactoglobulin

- Member of **Lipocalin** family - 14 members
- Function:
- Various binding functions:
- Retinol Prostaglandins
- Fatty acids Biliverdin
- Odorants Histamine
- Steroids
- Biochim. Biophys. Acta (2000). Special Issue

Equine β-Lactoglobulin

Two β-Lg's in equine milk, I and II

β-Lg I, 162 AA

β-Lg II, 163 AA, 1 extra AA at 117

Both I and II are monomeric

(i.e., no pH-dependent dimerization)

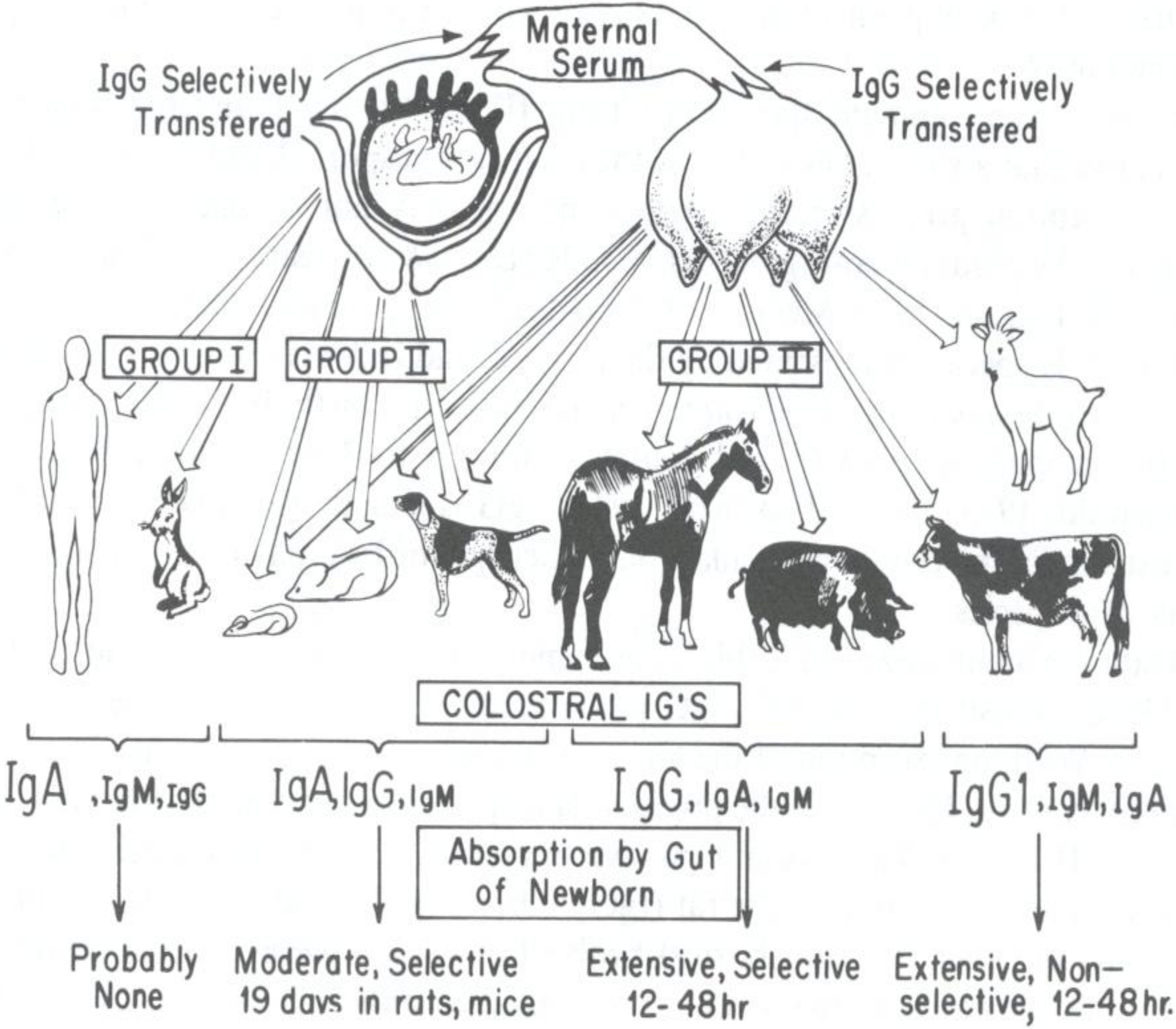
Both I and II have 2 intra-molecular

disulphide bonds

No sulphydryl

Equine Immunoglobulins (mg/mL)

Type	Human	Equine	Bovine	
	Colostrum	Colostrum	Milk	Colostrum
IgG	0.43	113.4	4.39	33-212
IgA	17.4	10.7	0.48	3.5
IgM	1.6	5.4	0.03	8.7



Maternal Serum
IgG Selectively Transfered
IgG Selectively Transfered
GROUP I
GROUP II
GROUP III
COLOSTRAL IG'S
IgA ,IgM,IgG
IgA,IgG,IgM
IgG,IgA,IgM
IgG1,IgM,IgA
Absorption by Gut of Newborn
Probably None
Moderate, Selective 19 days in rats, mice
Extensive, Selective 12-48hr
Extensive, Non-selective, 12-48hr.

Lactoferrin

- Non-haem iron-binding protein
- Present in milk, saliva, tears, bile, etc.
- Like seroferrin and ovoferrin
- MW: ~ 78,000 Da
- Primary and higher structures known
- Biological function:

    antibacterial, iron carrier, etc.

Equine milk relatively rich in Lf – 0.2-2 g/kg

i.e., 10x higher than bovine milk, slightly lower than human milk

(Doreau & Martin-Rosset, EDS)

Lysozyme (EC 3.1.2.17)

- Bactericidal
- 129 AA residues; MW ~14 kDa
- pI ~ 9
- Highly homologous to α-La – gene duplication?
- Human and equine milk – 3,000 and 6,000 more lysozyme than bovine milk
- Equine milk: ~ 800 mg Lys / L; 3% of total protein, ~7% of WP
- Human milk: ~500 mg Lys / L; 4% of total protein
- Equine milk is very shelf-stable – due to lysozyme?
- α-La binds and is stabilized by $Ca^{2+;}$

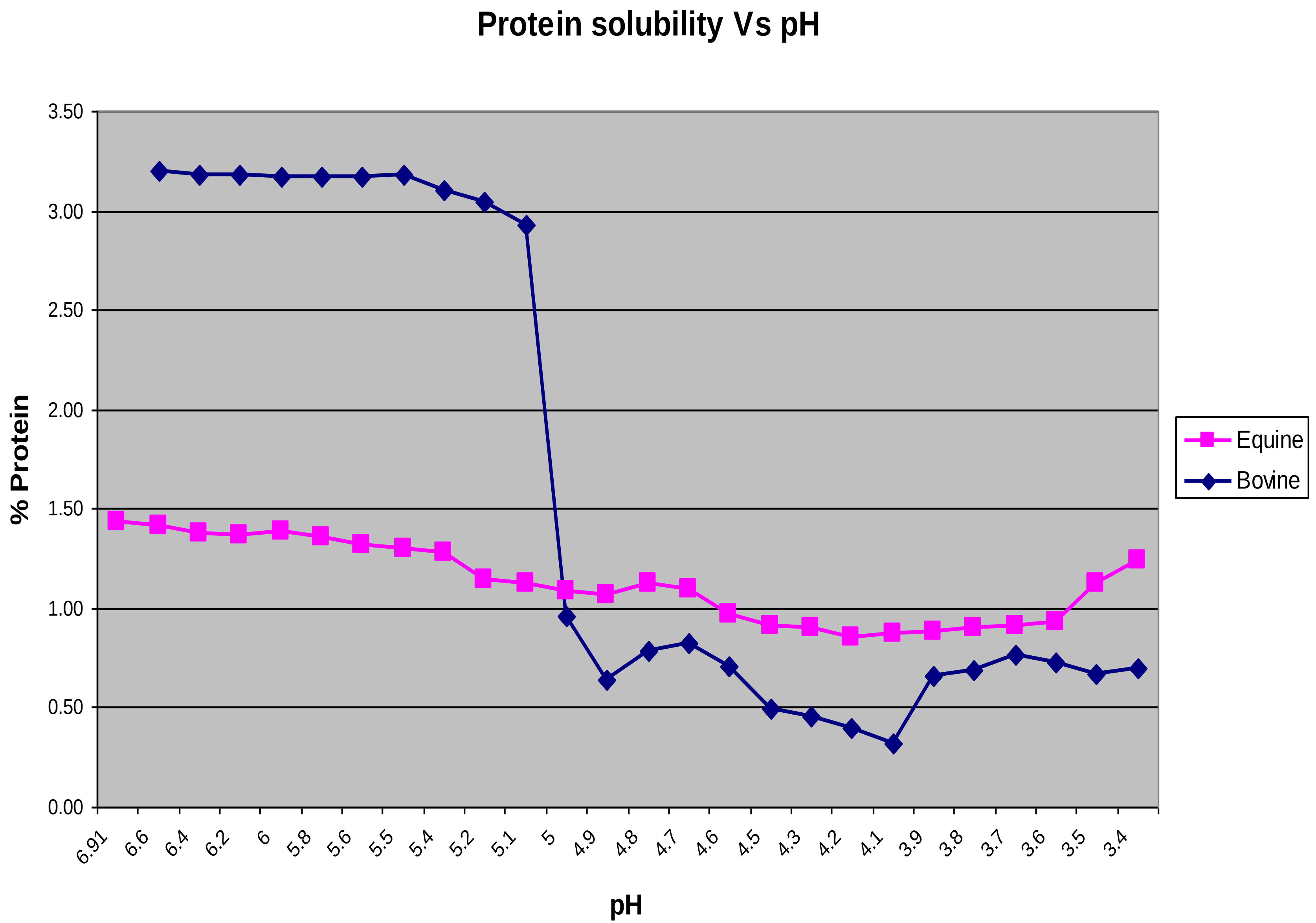
  lysozyme no - except equine lysozyme

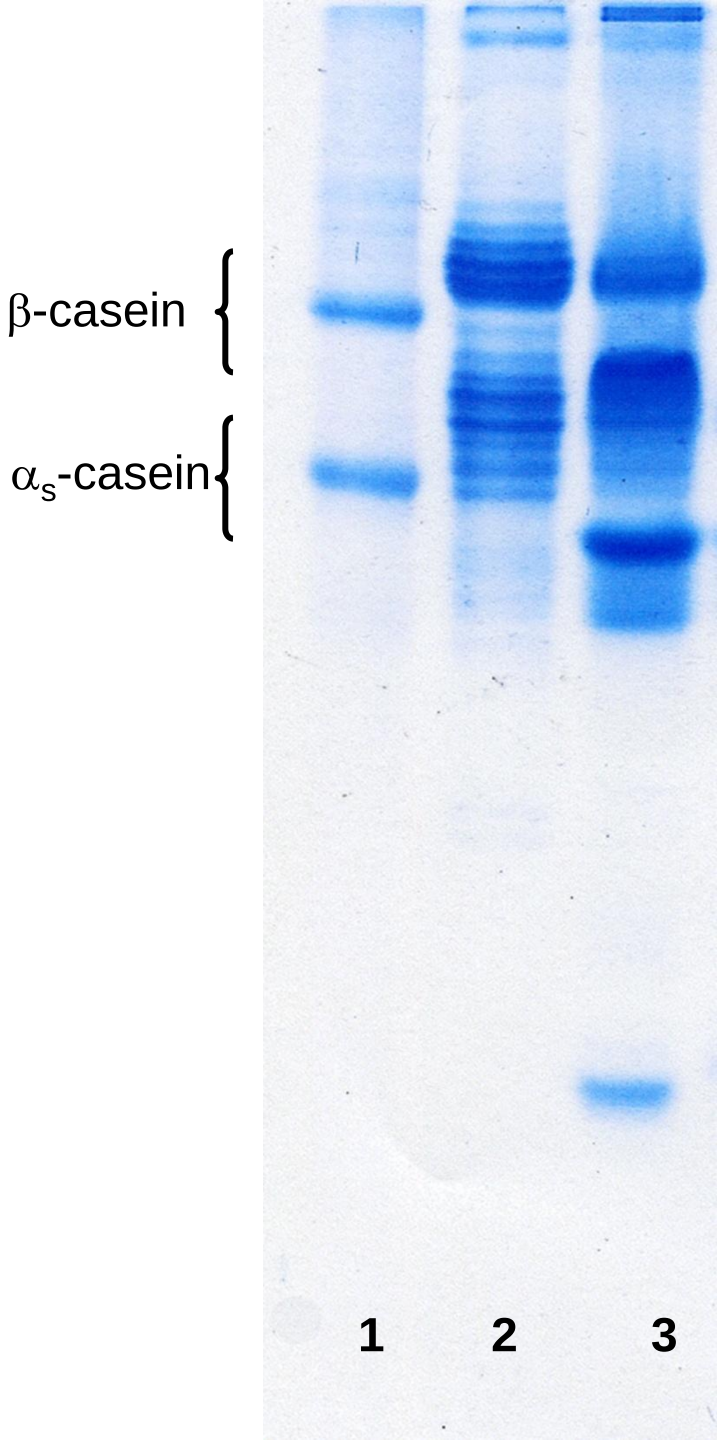
Indigenous Enzymes in Equine Milk

- About 70 enzymes reported in bovine milk
  (see Fox & Kelly, 2006a,b)
- Probably similar profile in equine milk → few reports
- Lysozyme: well studied
- Others:
  - Lactoperoxidase, catalase, amylase, lipoprotein lipase, plasmin, lactic dehydrogenase, malic dehydrogenase
  - Reported not to contain xanthine oxidoreductase –
  unlikely – export of fat globules
  - No reports on alkaline phosphatase, acid phosphatase, ribonuclase, N-acetlyglucosaminidase

Equine Caseins

- Very low casein content: ~1%
- About equal amounts of α_s- and β-caseins
- Both α_s- and β-caseins multiphosphorylated isoforms
  6 or 7 PO_4 residues per mol
- Very little κ-casein
- Amino acid sequence of α-, β- and κ- known


Protein solubility Vs pH
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
% Protein
6.91
6.6
6.4
6.2
6
5.8
5.6
5.5
5.4
5.2
5.1
5
4.9
4.8
4.7
4.6
4.5
4.3
4.2
4.1
3.9
3.8
3.7
3.6
3.5
3.4
pH
Equine
Bovine



1 Bovine Caseinate
2 Equine Caseinate
3 Equine milk

Our search for equine κ-casein

1. 2D electrophoresis, pH 4-7 and pH 3-10 followed by nano-LC MS-MS on ~ 64 spots from control and renneted equine milk – no κ-casein identified

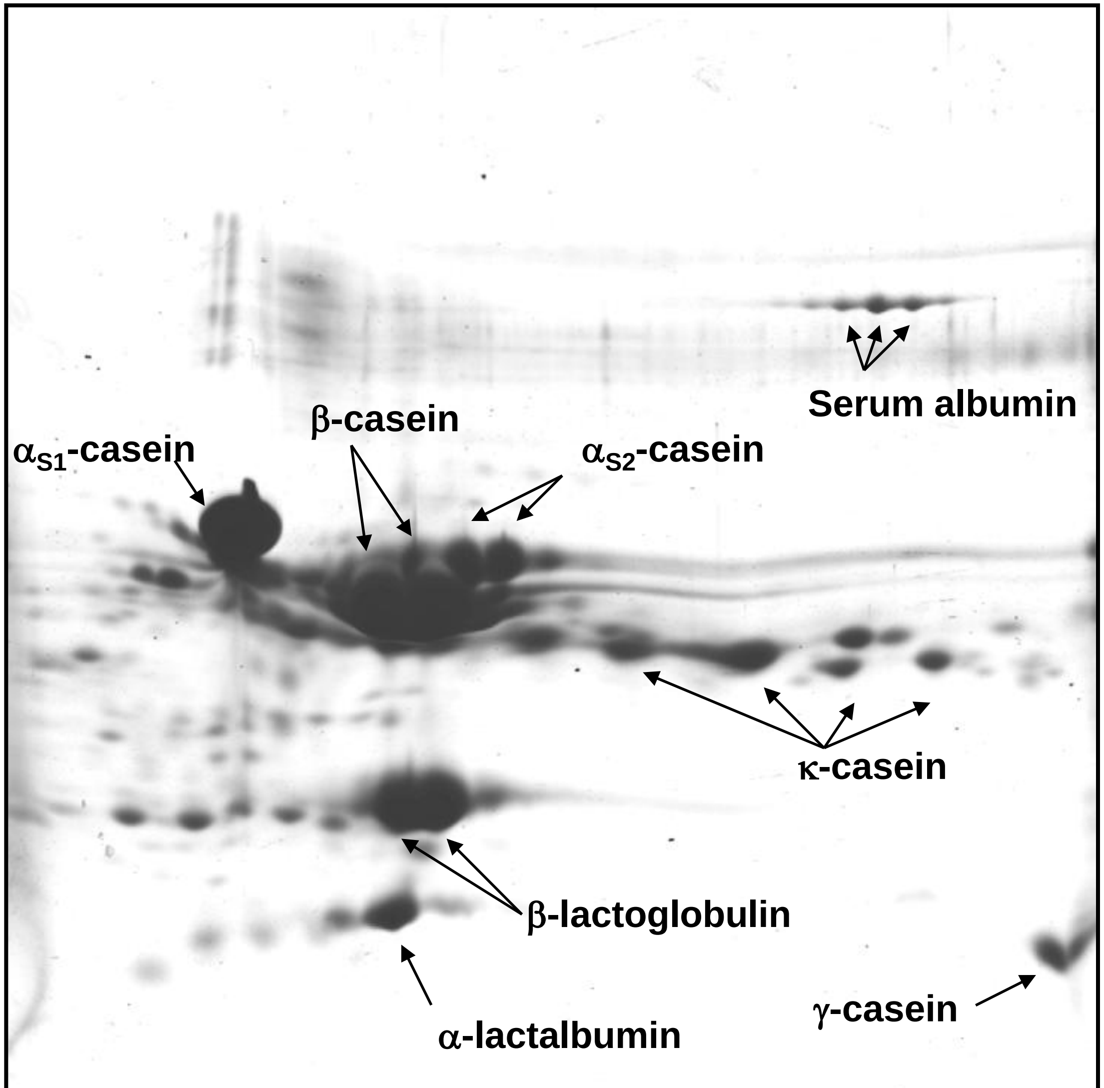
2. C18 RP-HPLC of 2% TCA fractions of renneted samples over 24 h – no CMP identified

3. C4 RP-HPLC of equine milk – on-going

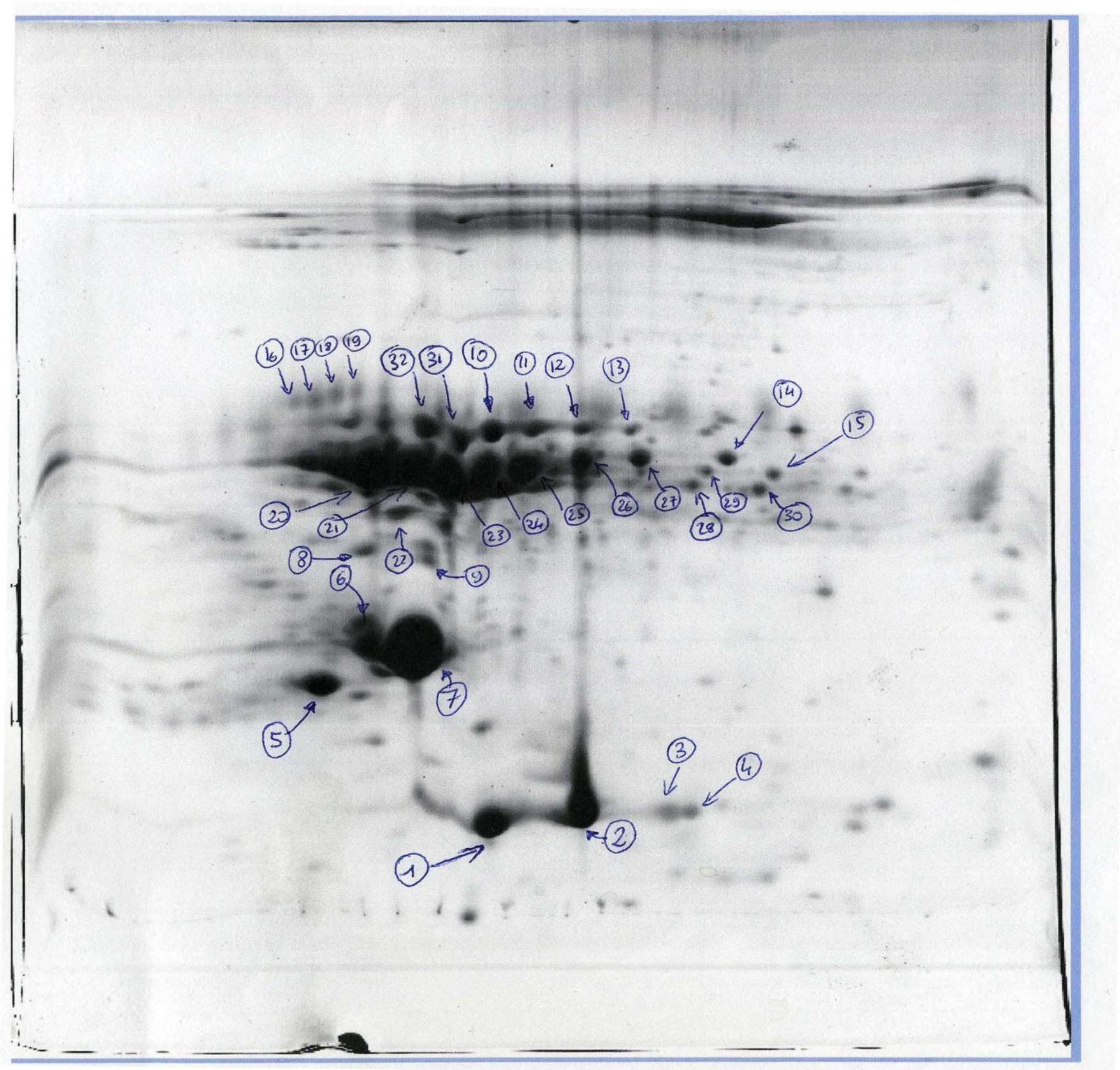
4. SDS gel electrophoresis with PAS – glycosylated protein present

5. Centrifugation of equine milk at increasing speeds followed by SDS PAGE – no κ-casein evident

2DE of bovine milk under reducing conditions
(strip 7 cm, pH 4-7, acrylamide gradient 12-18%)



2D Electrophoresis of Equine Milk – spots analysed by nanoLC-MS-MS



<u>Spots</u>
1-4, α-La
5-7, β-Lg
10-13, β-Cn
16-19, β-Cn
20-28, α_{s1}-Cn
30, α_{s1}-Cn
31, IgG
32, α_{s1}-Cn

pH 4-7 strips

Equine casein micelles

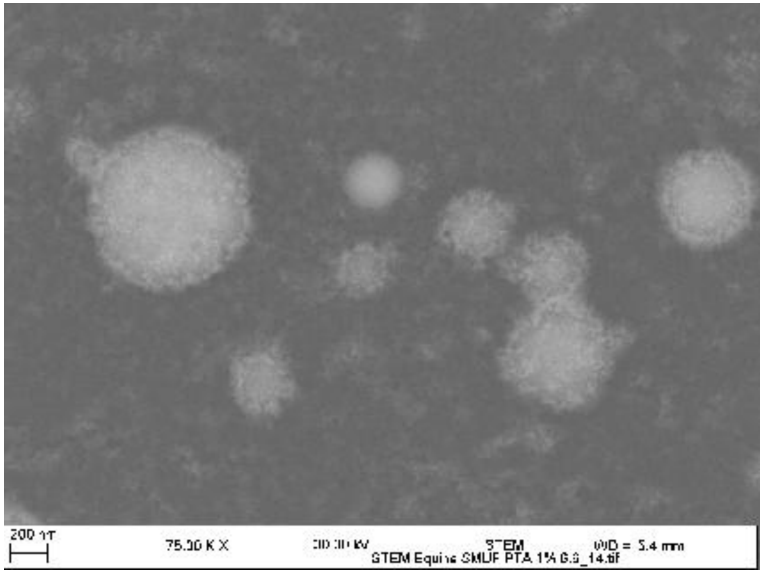
Equine casein occurs as micelles

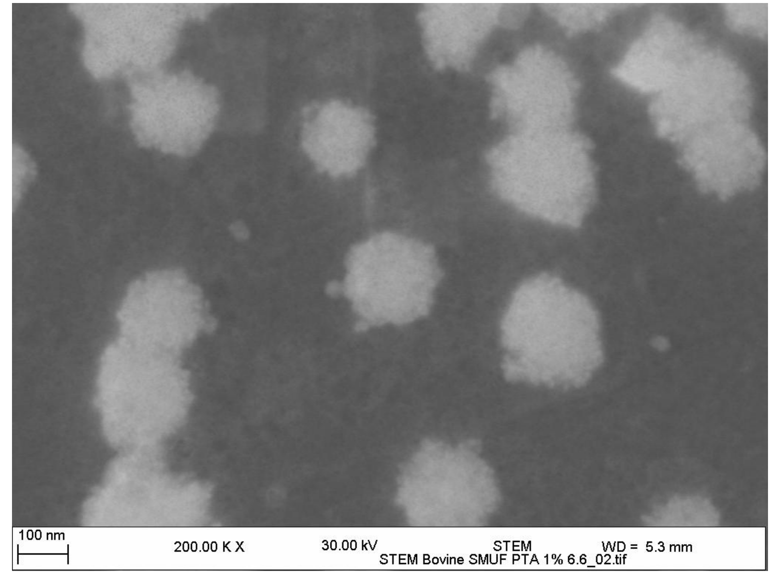
Generally similar to bovine casein micelles but slightly larger: average diameter of 275nm, vs 150nm

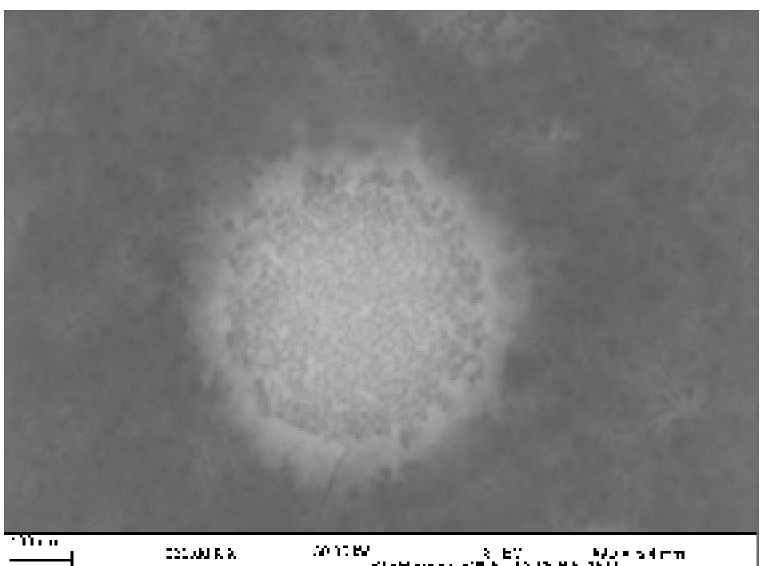
Zeta potential; lower than bovine at
-10mV vs -20mV

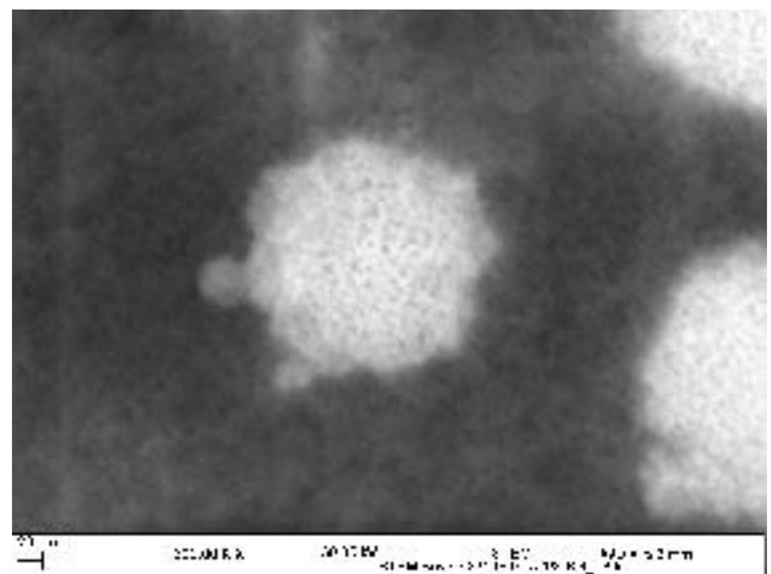
Ratio of micellar Ca:micellar inorganic phosphorus of 2.0 compared to 3.9 in bovine micelles

STEM of equine and bovine micelles

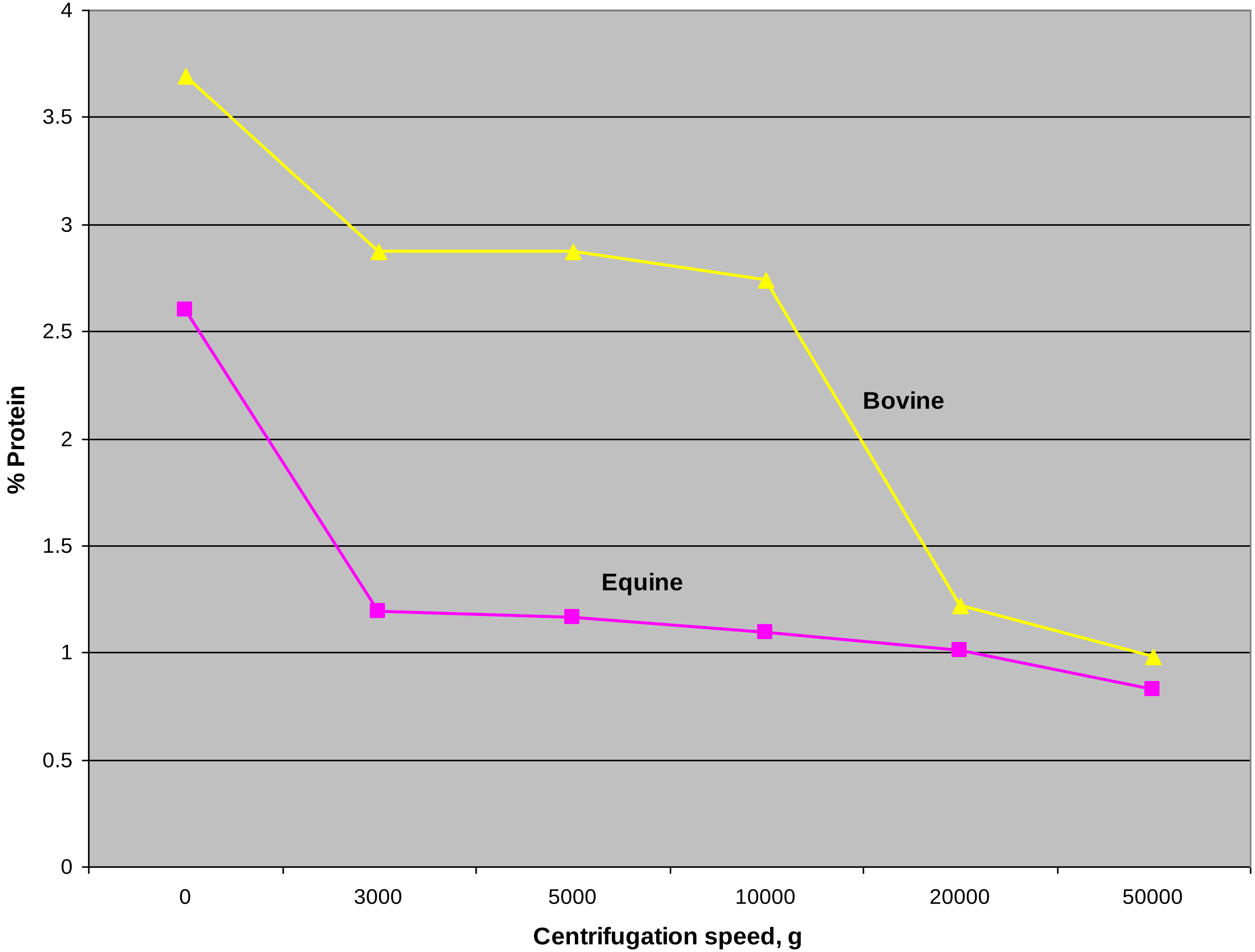








% Protein in supernatant vs centrifugation speed for equine and bovine milks



Stability of Equine Micelles

- Heat
- Acidification
- Renneting
- Ethanol
- Urea
- Ca-chelators
- Low temperature

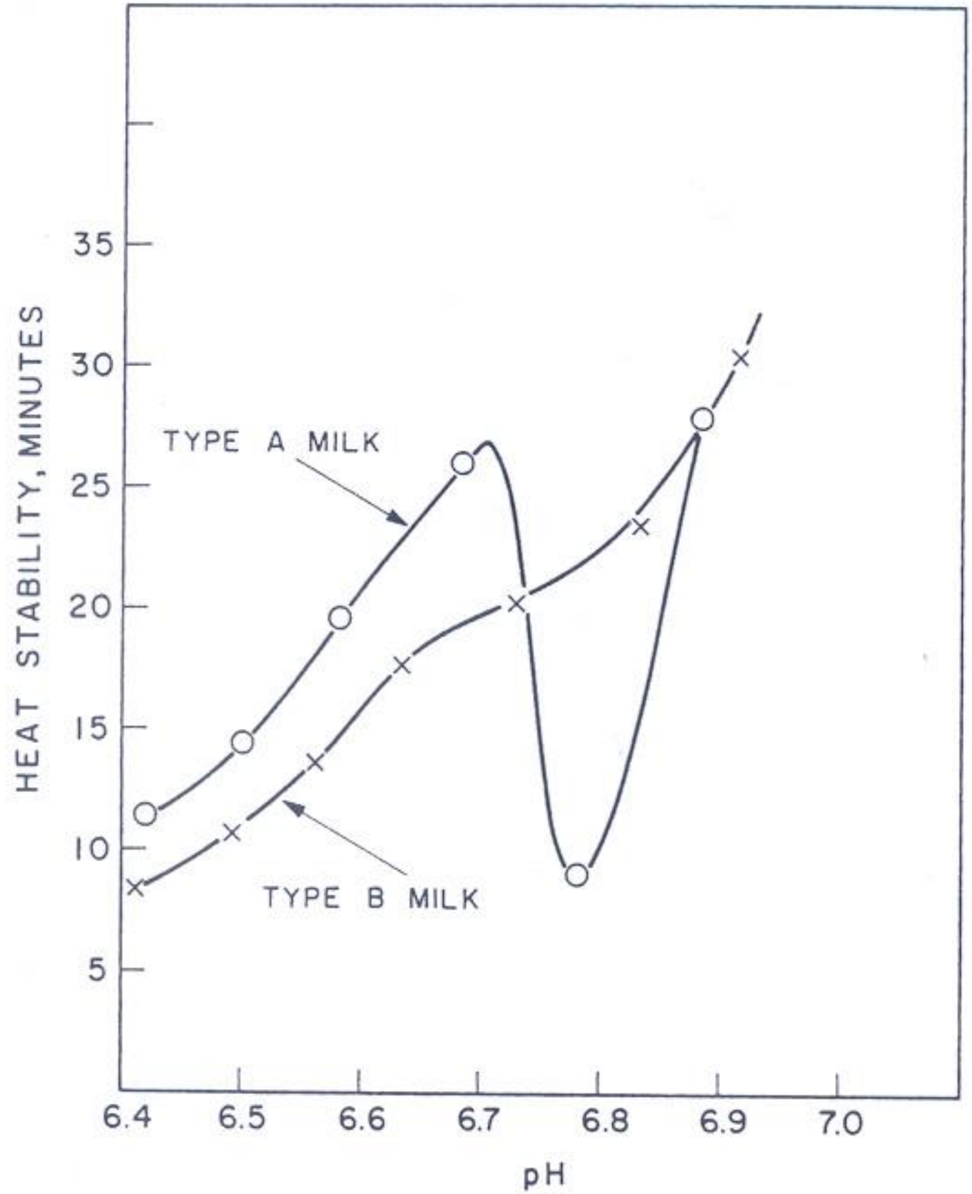
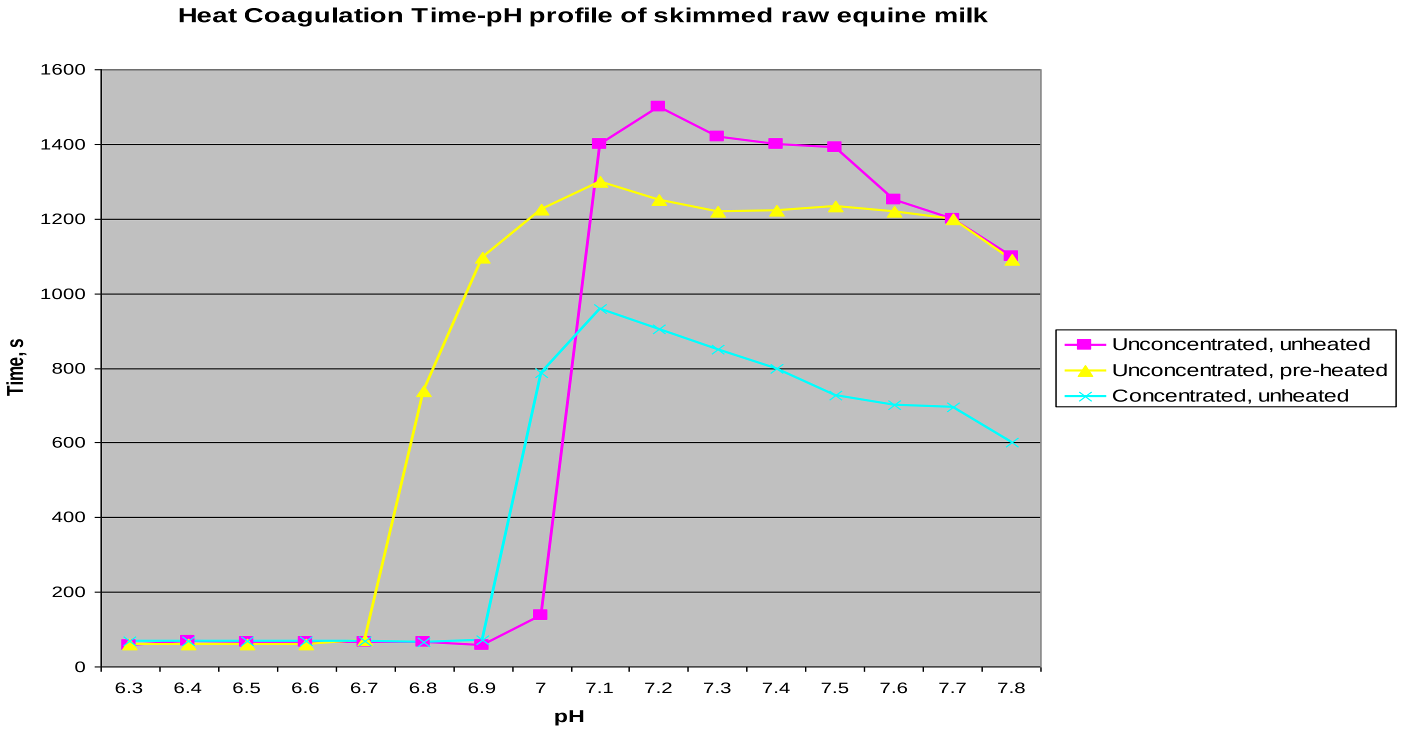


FIG. 1. pH–heat stability curves of individual milks. ●—Type A milk; ×—Type B milk.

Stability of Equine Casein Micelles

- <u>Heat Stability</u>
- heat stability very low (< 2 min at 140ºC)



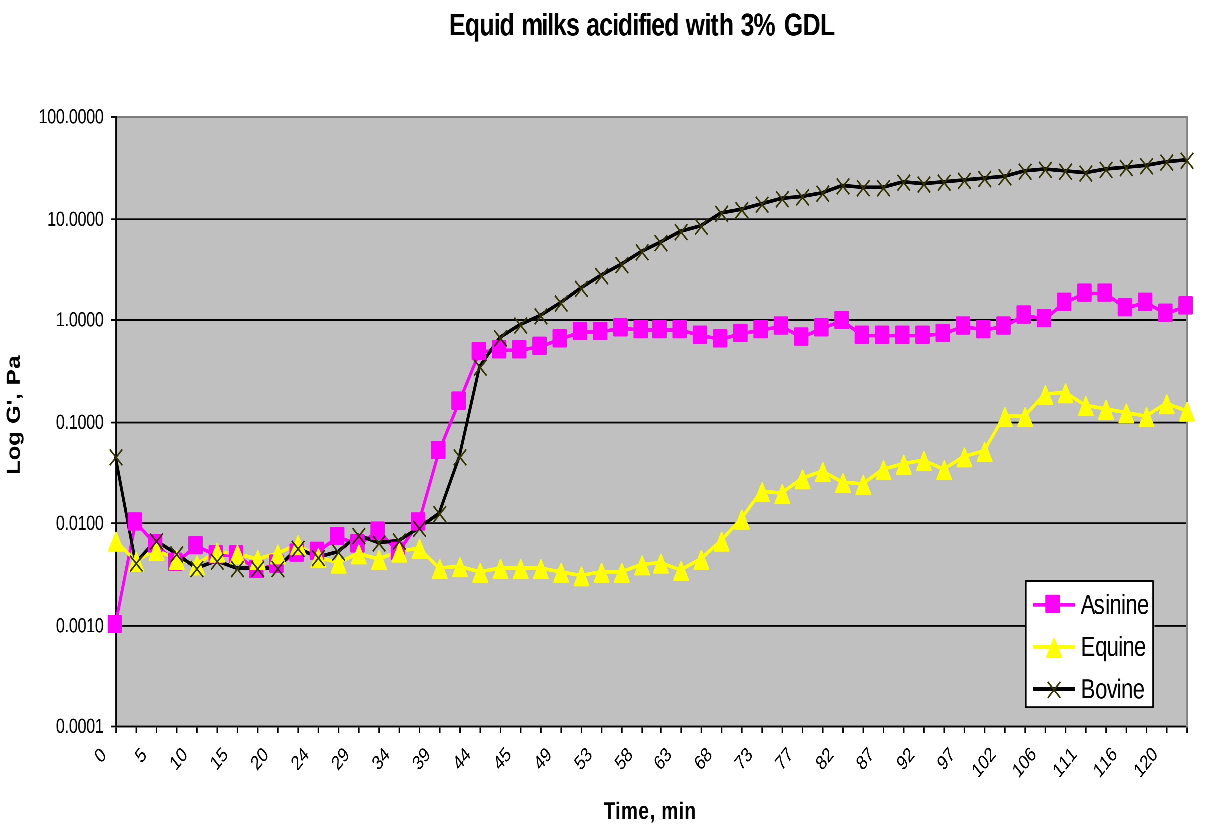
Type A HCT-pH profile

High [Ca^{2+}] may be responsible, i.e 2.6mM

Very protracted clotting with pre-heated equine milk

Stability of Equine Casein Micelles

Acid-induced coagulation

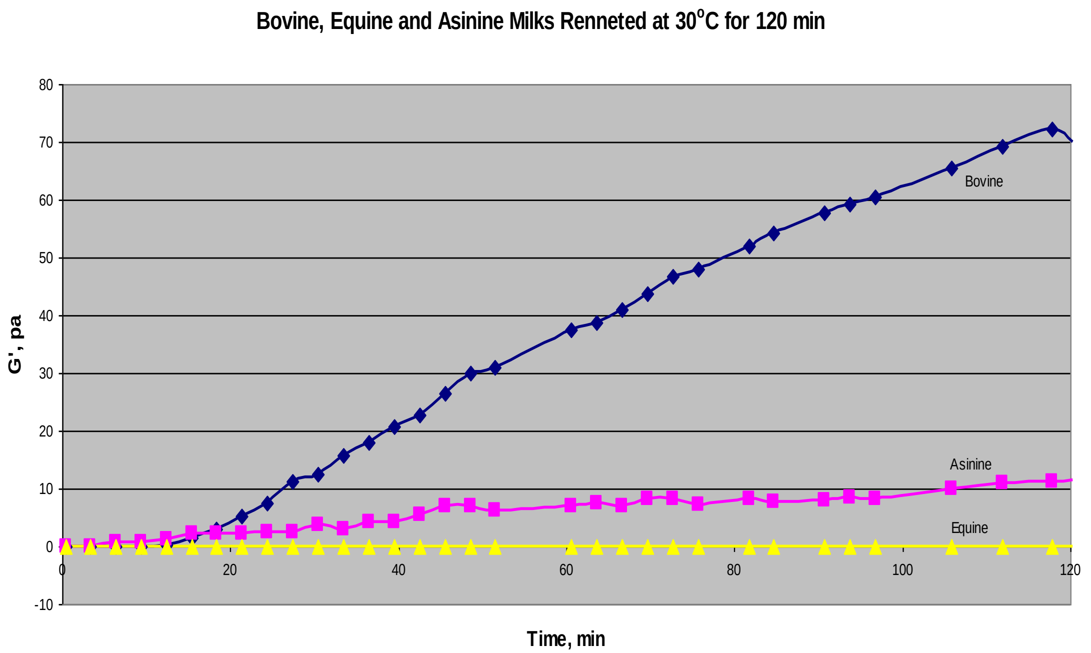


At pH 4.2 approx. precipitate forms but no gel, increasing casein content or slowing rate of acidification has little effect

Stability of Equine Casein Micelles

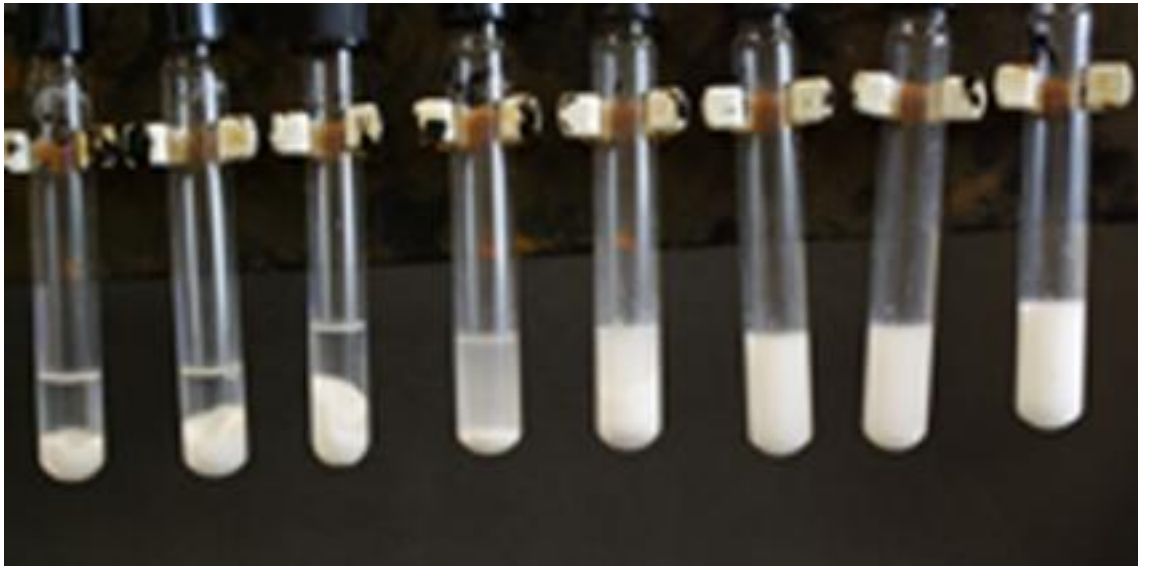
Rennet-induced coagulation

– no coagulation visually, no G' increase detected



- no cheese produced from equine milk
- no gel at 2X concentration of equine protein

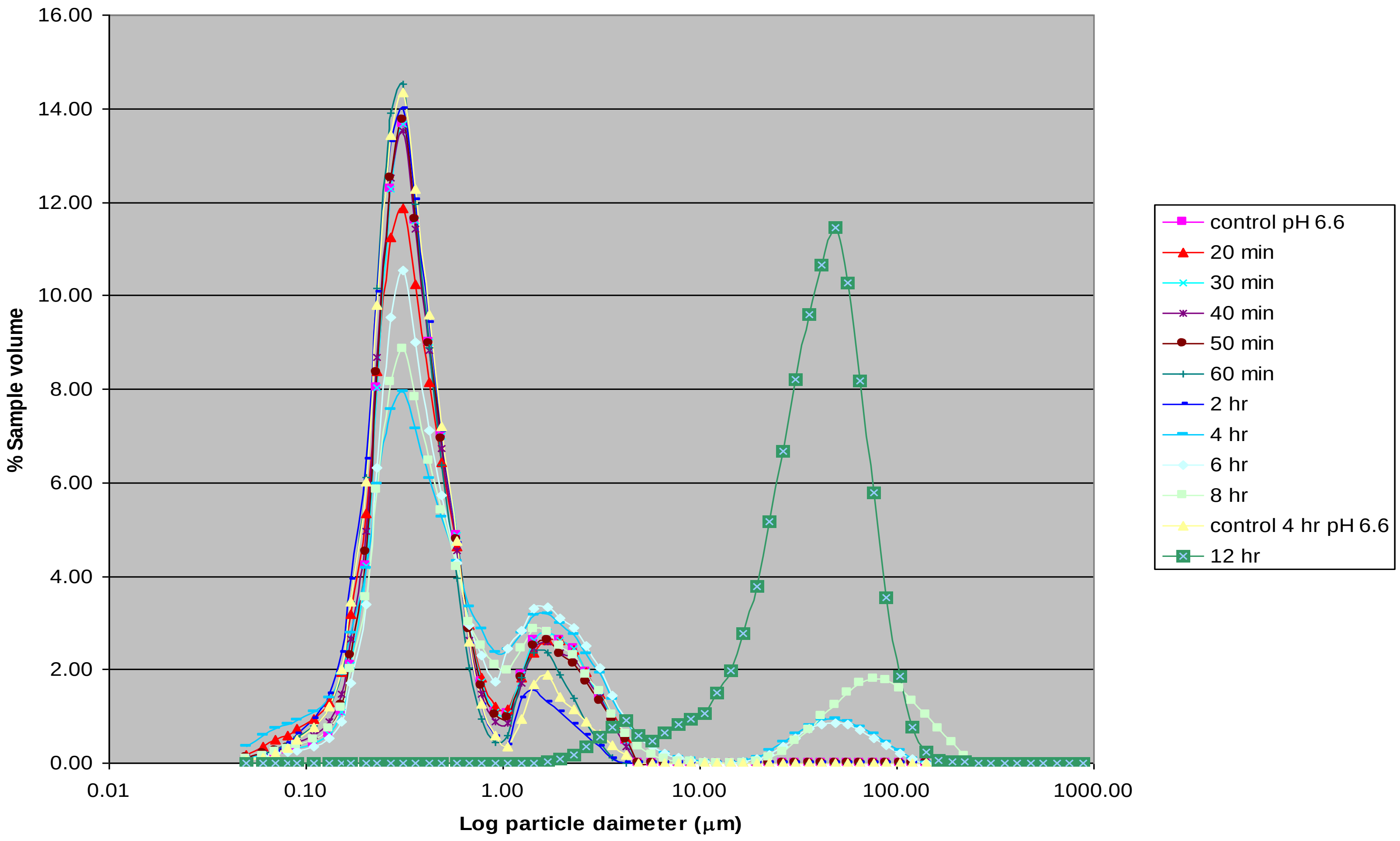
Equine milk renneted at different pH values



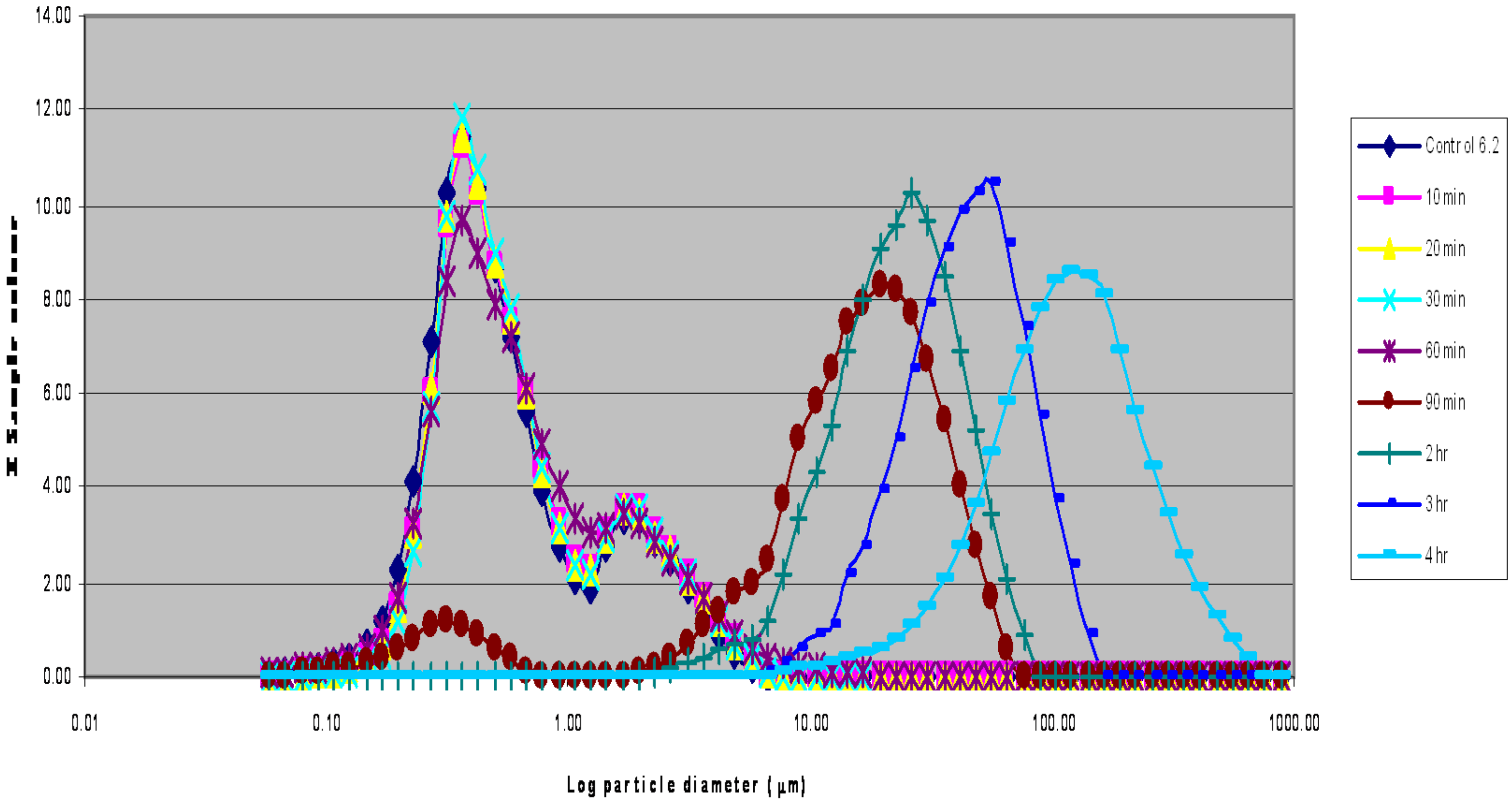
Left to right: pH 6.0, 6.1, 6.2, 6.3, 6.4, 6.5, 6.6 and 6.7

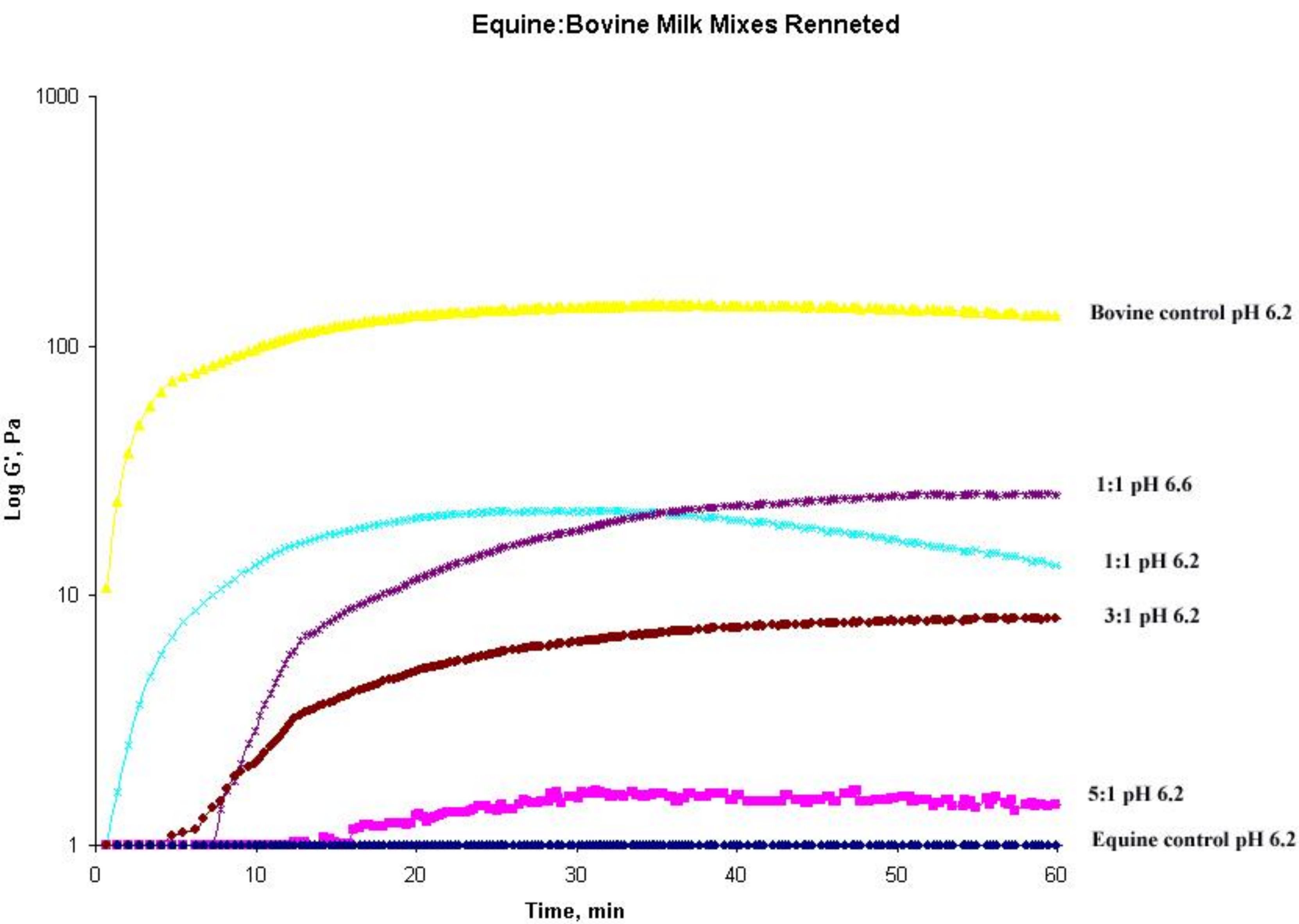
No curd formation but at lower pH values the milk forms floccs

Aggregation of renneted equine milk at pH 6.6 assayed by laser light scattering



Aggregation of renneted equine milk at pH 6.2 assayed by laser light scattering





Equine:Bovine Milk Mixes Renneted
1000
100
10
1
0
10
20
30
40
50
60
Log G', Pa
Time, min
Bovine control pH 6.2
1:1 pH 6.6
1:1 pH 6.2
3:1 pH 6.2
5:1 pH 6.2
Equine control pH 6.2

Renneting of Mixtures of Bovine and Equine Milks

- PAGE of whey and curd from renneted mixtures of bovine and equine milk
- Showed that at least some equine caseins incorporated into curd
- More work required

Stability of Equine Casein Micelles

Alcohol stability

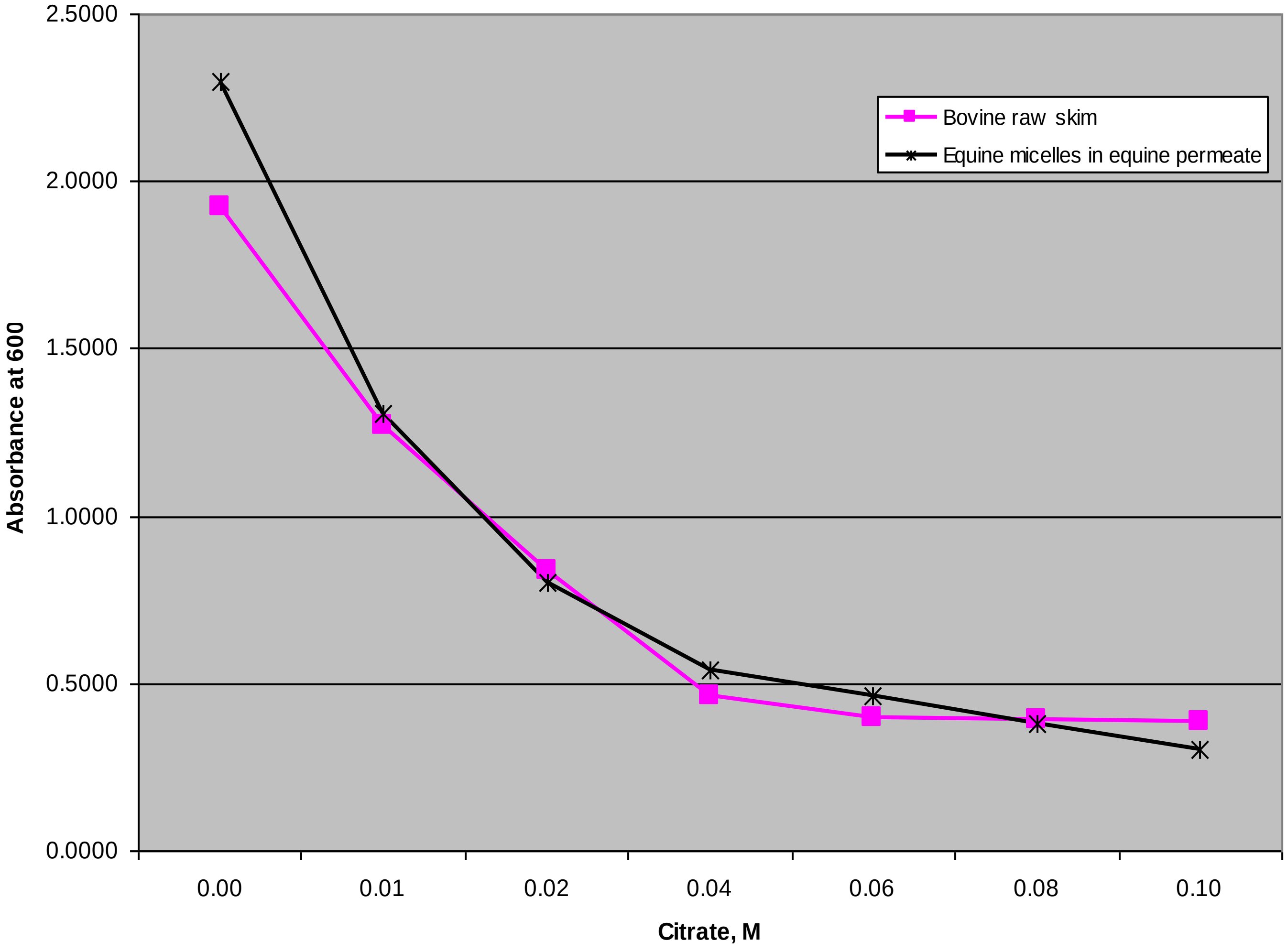
- At natural pH, equine milk stable to ~ 45% aqueous ethanol (v/v) [about same as bovine milk]

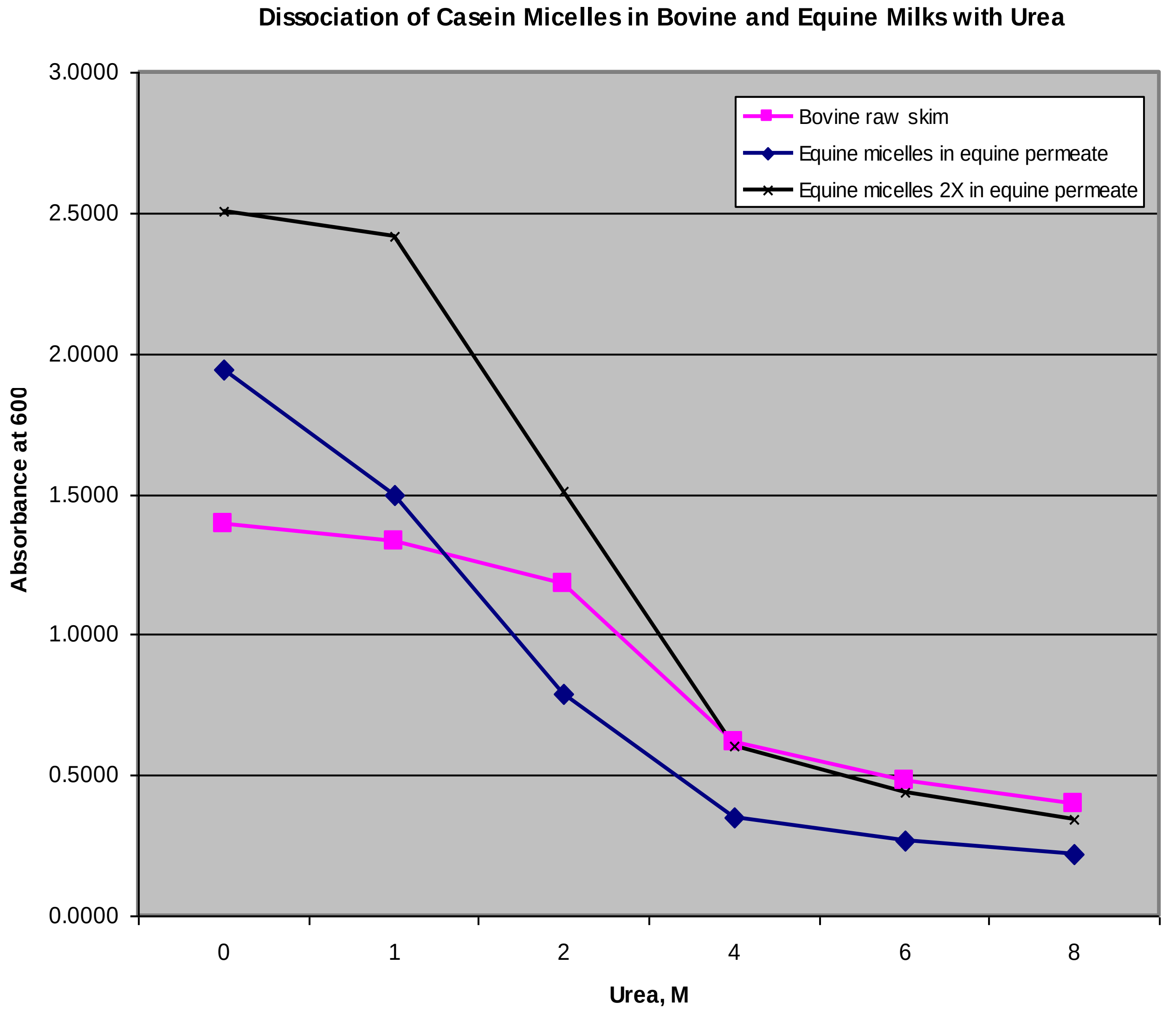
- Ethanol-mediated temperature-induced dissociation of casein micelles very different to bovine milk

- Addition of 70% v/v aqueous ethanol to bovine milk and heating to 70^{o}C – becomes translucent. Cooling bovine/alcohol mix to 20^{o}C or removal of ethanol restores of 'milky' appearance.
- Equine milk-ethanol not dissociated.

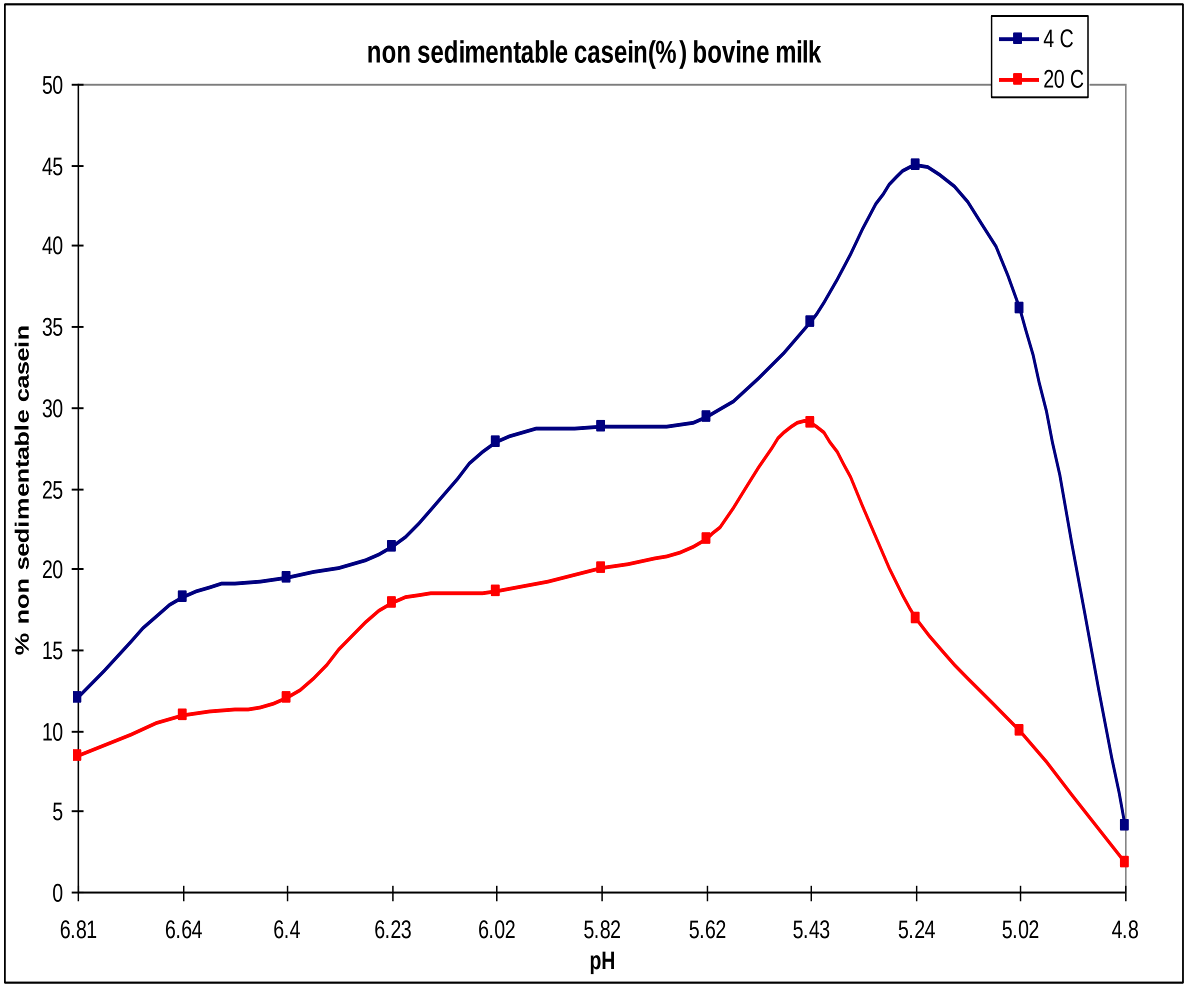
- Addition of 70% v/v aqueous ethanol to bovine milk, heat to 70^{o}C, cool on ice $\Rightarrow$ gelation; not equine milk

Dissociation of Casein Micelles in Bovine and Equine Milks with Citrate

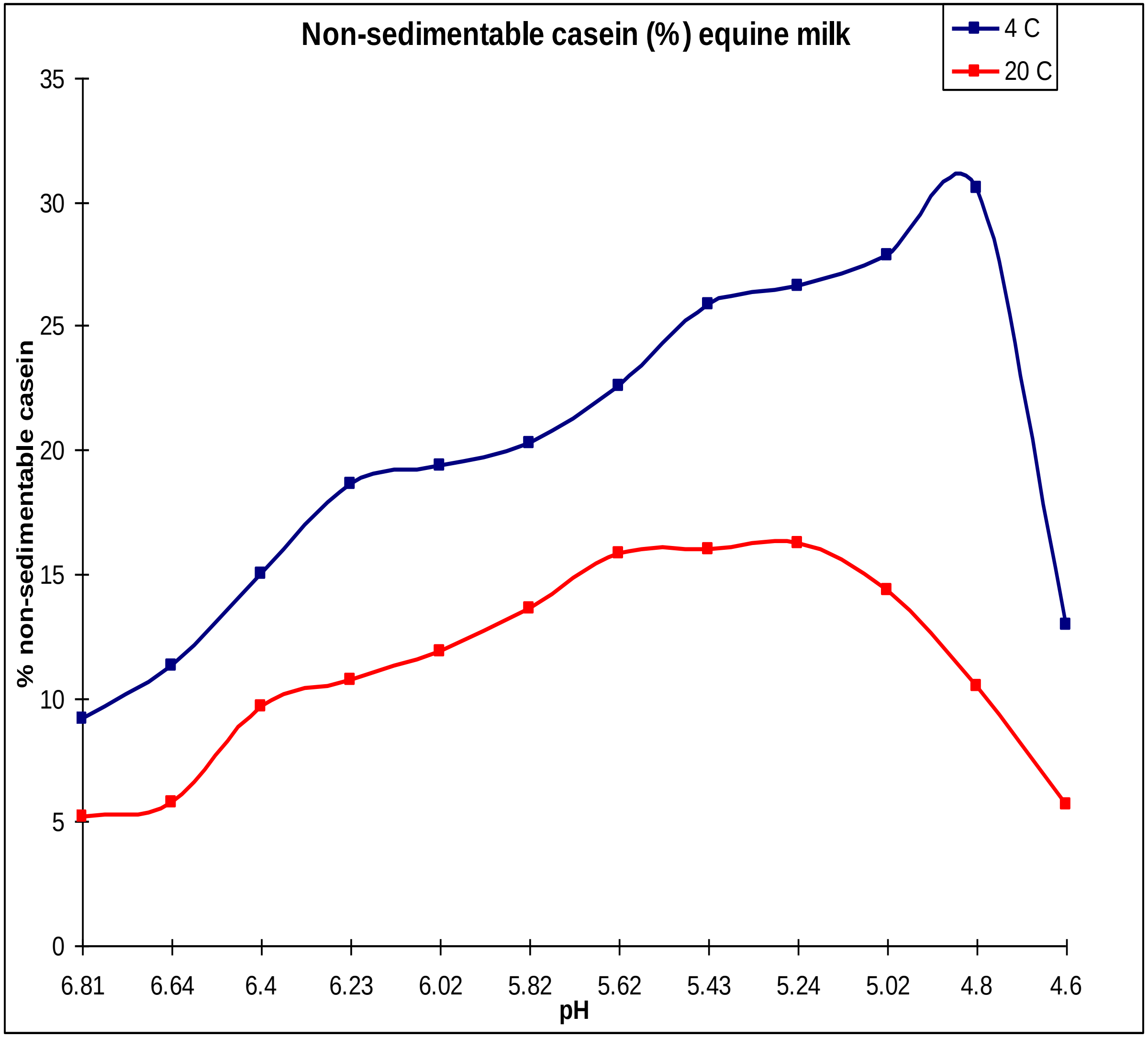



Dissociation of Casein Micelles in Bovine and Equine Milks with Urea
Absorbance at 600
3.0000
2.5000
2.0000
1.5000
1.0000
0.5000
0.0000
0
1
2
4
6
8
Urea, M
Bovine raw skim
Equine micelles in equine permeate
Equine micelles 2X in equine permeate

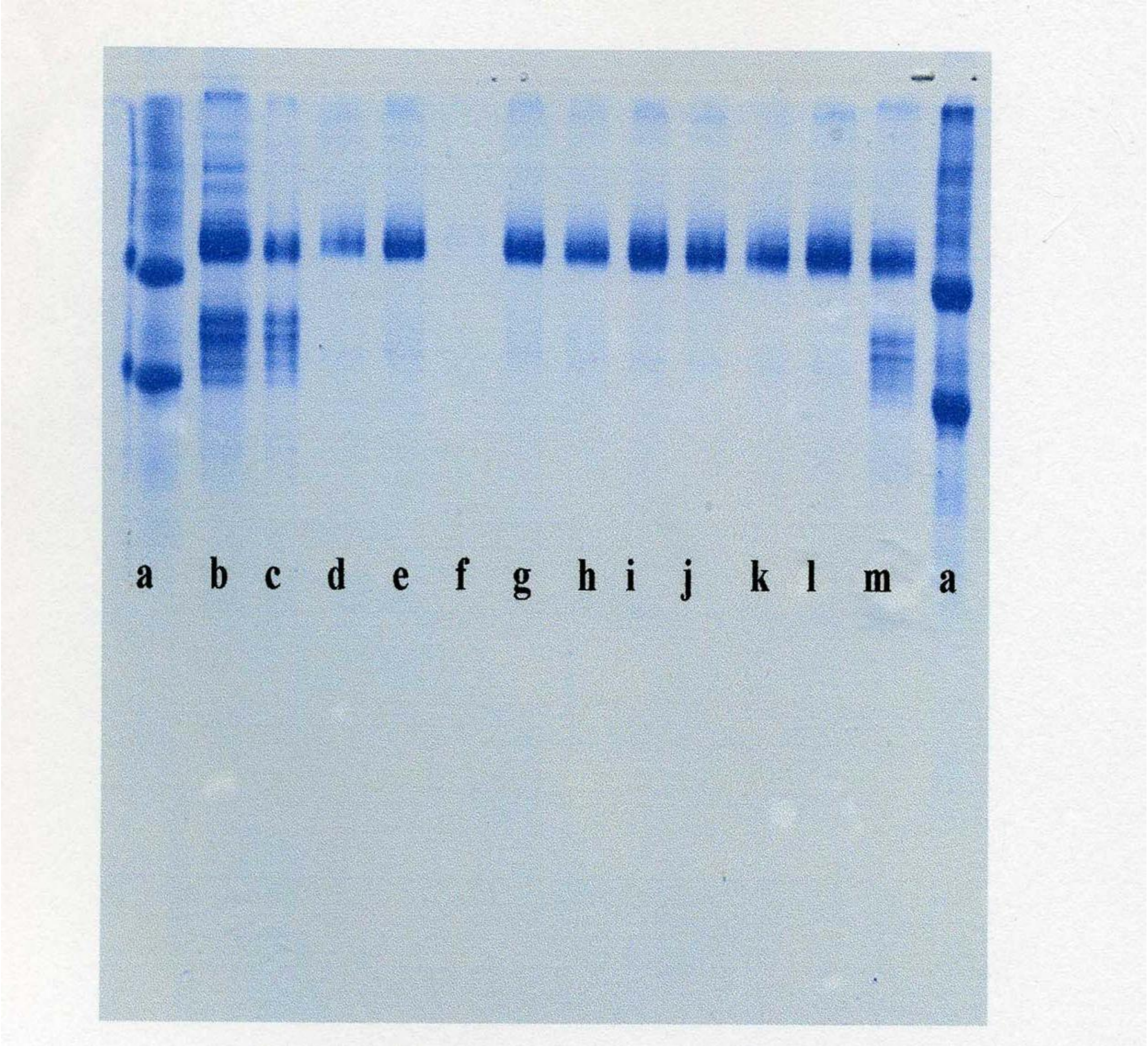
Non-sedimentable (70,000 g x 2 h at 20^{o}C or x 4 h at 4^{o}C) casein in bovine milk,



Non-sedimentable (70,000 g x 2 h at 20°C or x 4 h at 4°C) casein in equine milk,



Non-sedimentable casein from equine milk at pH 6.7(d) to 4 (l)



Consumption of Equine Milk

- **Historical aspects**
  - traced back to 2000BC. Central Asia, Russia, Eastern Europe

- **Current trends**
  - 30 million people worldwide drink equine milk regularly
  - potential for further use in dietetics and therapeutics
  - used for premature infants (composition similar to human milk etc, easily digested)
  - used in the diets of the elderly and convalescents

- **Health-giving claims**
  - using equine milk to treat many ailments [lack of epidemiological studies] tuberculosis (Russia), hepatitis, peptic ulceration, children with BMA
  - low fat, low cholesterol, exceptionally high PUFA's (ω-3)
  - Probiotic and prebiotic effects
  - antibacterial effects due to high lysozyme and lactoferrin
  - Suggested dosage is generally about 250 mL equine milk/day

Equid Milk Products

Pule – Balkan donkey milk cheese ~ €1,000/kg! Donkey milk products – Zasavica, Serbia

Koumiss

Horse milk products

Equine Milk Literature

General reviews:

Doreau, M (1994) *Lait* 74, 401-418

Doreau M & Martin-Rosset, W (2003), *EDS*,

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Park, Y.W. *et al.* (2006). Mare milk, in,

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<u>Equid milks:</u>

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